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14. ABSTRACT

The objective of this study is to develop models to causatively link Spot 14 (S14) expression in the mammary epithelium and tumor formation, metabolism, and metastasis. Our hypothesis is that S14 promotes de novo lipid biosynthesis in tumor cells, causing enhanced proliferation and ultimately, tumor metastasis. Using a genetic model of spontaneous tumorigenesis, we have found that the presence of S14 significantly accelerates tumor latency, and is associated with branching and budding of the non tumor- bearing mammary glands. Additionally, we have established cell lines from S14-/- mammary epithelium, transformed with the Polyoma Middle T antigen (PyMT) or Neu oncogenes. These lines will be used in xenotransplant experiments to test the requirement of S14 for mammary tumor growth and cellular lipid metabolism.

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mammary tumor, breast cancer, Spot 14, lipid synthesis

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Table of Contents

Page

Introduction **4**

Body **5**

Key Research Accomplishments **10**

Reportable Outcomes **11**

Conclusion **13**

References **15**

Supporting Data **16**

Introduction

Since 1920, it has been recognized that the metabolism of cancer cells differs from normal cells in the rate of glucose transport, and that tumors display an elevated rate of aerobic glycolysis. Less well appreciated are alterations in lipid metabolism and the high rates of de novo fatty acid biosynthesis displayed by many tumors (1). While regulation of anabolic metabolism (i.e. lipid biosynthesis) is important to normal mammary epithelial function during lactation, breast cancers that displaying elevated lipogenesis correlates with a reduced disease free survival (2). Two related prognostic indicators are expression of fatty acid synthase (FASN) and thyroid hormone responsive protein Spot14 (S14) (3). Much is understood regarding the regulation and function of FASN in tumor cells, but little is known about the contribution of S14 to the lipogenic phenotype. Existing data suggests expression of S14 and/or elevated lipid biosynthesis in breast tumors correlates with a poor clinical prognosis, however there is no clear demonstration of a causal relationship between S14 and lipid biosynthesis in tumor cells. Furthermore, the behavior of tumors with elevated S14 and lipid biosynthesis is not well characterized in vivo with regard to rate of tumor growth, rate of tumor metastasis, and alterations in tumor metabolism. Demonstration that S14 can stimulate lipid biosynthesis in breast cancer cells, or enhance tumor growth and/or metastasis will establish a role for S14 and lipid biosynthesis in these processes. The objective of this study is to develop models that will reveal a causative link between S14 and tumor growth, metabolism and metastasis. We hypothesize that S14 expression will promote de novo lipid biosynthesis in tumor cells, thereby facilitating tumor growth and metabolism. We propose to test our hypothesis using models of mammary tumorigenesis induced by ErbB2/Neu, Polyomavirus Middle T antigen (PyMT), or Ras oncogenes. In addition, we propose to evaluate the effect of S14 loss and overexpression on the mRNA levels of genes encoding enzymes that play roles in various metabolic pathways to begin uncovering the mechanism of S14 action.

Body

From SOW: Specific Aim 1*: Determine the effect of Spot14 loss on the growth and metabolism of tumor cells expressing Neu, PyMT or Ras oncogenes.*

Task 1 (Year 1 Months 1-3): We will isolate primary mammary epithelial cells from mid-pregnant Spot14-/- and C57Bl/6 WT mice. These cells will be transformed with retroviruses endoding Neu, Ras, or the Polyoma Middle-T antigen (PyMT). The cells will be selected using the appropriate drugs and transformed lines will be established and preserved. We anticipate completing this task the first quarter of the first year.

The objective of this Specific Aim is to determine the requirement of S14 for cancer cell proliferation in vivo, and the effect of S14 loss on tumor lipid metabolism. In the first quarter of Year 1, we isolated primary mammary epithelial cells from S14-/- and C57Bl/6 WT mice. These lines were transformed with the PyMT oncogene, and were established as proliferating populations. The final approval from ACURO to perform xeno-transplant experiments using these cell lines was received on July 7, 2011; therefore, these studies have been pushed back to this fall so that I may use the allocated DoD funds for this project. Because of this, Tasks 2 and 3 of Specific Aim 1 have not been completed. This is only a minor set-back, though, because I discovered something very important about my cell line models and am able to modify my experimental design before performing any transplant surgeries, saving money and time in the long run. I believe the modifications, explained below, will result in a more appropriate model than I had originally proposed.

After establishing proliferating populations of S14-/- and WT mammary epithelial cells, I began characterization of the cells to ensure their epithelial origin. I discovered that cells from S14-/- mice expressed robust cytokeratin 18 (CK18), a routinely used marker of epithelial cells, but the cells from WT mice did not express CK18. When I examined expression of CK18 in very early (passage 2) WT mammary epithelial cells, I found that these cells *did* express CK18, which told me that as the cells transformed and expanded, they lost their epithelial characteristics. It is possible that using both the CK18-positive S14-/- cells and CK18-negative WT cells in xeno-transplant experiments would result in different outgrowth patterns that would not reflect the absence of S14, but would instead reflect the different cell type of origin (epithelial versus mesenchymal). This could lead to inappropriate data interpretation and could cause me to draw the wrong conclusion about the role of S14 in tumor growth. Based on this difference in cell types, I chose to alter the model slightly. Instead of using the cells from WT C57Bl/6 mice as a control, I have begun the process of re-expressing S14 in the S14-/- cells, and will use those cells as a control for my experiments. This way, I will be sure that the cells are virtually identical except for the presence of S14. The difference in cellular characteristics between S14-/- and WT mice was not anticipated when I submitted the original proposal. To achieve re-expression of S14, I initially chose to use the Tet-On model of gene expression. This relies on stably expressing the rTTA sequence in the target cell line, followed by stable expression of the gene of interest (S14) regulated by

the Tetracycline responsive promoter. When tetracycline or doxycycline is added to the target cell population, the gene of interest will be induced. We have used this system in various other cancer and normal cell models in our laboratory. I have had some trouble expressing the rTTA sequence in the target cells, and the explanation for this is not clear. To overcome this problem, I am in the process of sub-cloning HA-tagged S14 into a stable expression vector and will directly express S14 in the S14-/- target cells, eliminating the need for the dual sequence expression. This method will be more simple and straightforward than the Tet-On system, and will guarantee constitutive expression of S14 in the tumors once they are established. The expected outcomes and study endpoints for this Specific Aim have not changed, but I believe that the adjusted model is more suited for testing the effect of S14 loss on tumor growth and metabolic characteristics.

Based on what I found with the PyMT-transformed mammary epithelial cells and the adjustments I made to that model, I chose to isolate primary mammary epithelial cells from S14-/- mice on the FVB background for the Neu-transformation studies. It was pointed out by reviewers of my proposal that cells from C57Bl/6 mice are often resistant to transformation by the Neu oncogene. Because of this possibility I chose to use the FVB background for this portion of the study. I have successfully isolated primary mammary epithelial cells from S14-/- FVB mice, have stably expressed the Neu oncogene, and have established proliferating populations of cells that are CK18 positive. As with the PyMT studies, I am in the process of re-expressing S14 in these cells as a control, rather than using WT cells from FVB mice. Again, the study endpoints and expected outcomes have not changed, but I believe the model is much improved.

Task 2 (Year 1 Months 3-6): The transformed Spot14-/- or WT cells will be injected into the fat pads of adult, nulliparous recipient mice. Tumor growth will be monitored twice weekly and the animals will be harvested when the tumors reach 0.5 cm in diameter. The tissue will be collected for protein, RNA, and metabolomic analyses according to the methods outlined in the Project Proposal. We anticipate completing this task the second quarter of the first year.

Task 3 (Year 1 Months 6-12): The preserved tumor samples collected from the recipient mice will be analyzed using qPCR, western blot, MRS, and immunohistochemistry. We anticipate completing this task by the end of the first year.

As described above, I did not receive ACURO approval until July 7, 2011, so I have not performed Task 2, which means I have no samples to use for Task 3 at this time. I was able to make adjustments to the model and I believe that in the long run, it will yield data that will be more informative and accurate as to the role of S14 in tumorigenesis.

***From SOW: Specific Aim 2**: Determine the effect of S14 overexpression on the onset, growth, metastasis and metabolism of mammary tumors arising in the MMTV-ErbB2 mice by generating MMTV—ErbB2, MMTV-Spot14 bitransgenic mice.*

Task 1 (Year 1 Months 1-6): We will breed the MMTV-Spot14 and MMTV-c-ErbB2 mice to generate the single and bi-transgenic offspring that will be used in our studies. We anticipate completing this task by the end of the second quarter of the first year.

The objective of this Specific Aim is to determine if S14 expression is sufficient to promote tumor formation, cell proliferation, and lipid synthesis in ErbB2 expressing mammary epithelial cells. During Year 1, we began breeding MMTV-S14 female mice with MMTV-c-ErbB2 male mice to establish colonies of bitransgenic (ErbB2/S14) and single transgenic (ErbB2) animals to use for tumor studies. Breeding is still ongoing for this study. To date, we have a total of 15 ErbB2/S14 and 10 ErbB2 mice in our study.

Task 2 (Year 1 Month 6-Year 2 Month 6): The MMTV-Spot14, MMTV-c-ErbB2, and MMTV-Spot14/MMTV-c-ErbB2 mice will be monitored for tumor onset and growth. The tumors will be evaluated as described in the project proposal and the animals will be sacrificed when the tumor reaches 0.5 cm in diameter. The tumor and other tissues will be harvested and preserved. We anticipate completing this aim in the second quarter of the second year.

During Year 1, we monitored existing animals for tumor onset by manual palpation twice-weekly beginning when the animals were 4 months old. To date, we have latency data for 15 ErbB2/S14 and 10 ErbB2 female mice. Our hypothesis was that S14 overexpression in this model would accelerate tumor formation (shortening the latency) and would promote tumor growth. We have observed a significantly shorter tumor latency in ErbB2/S14 mice compared to the ErbB2 controls (Figure 1), supporting the hypothesis that the presence of S14 in tumors promotes tumor formation. It has been demonstrated in human tumor samples that S14 and FASN are directly correlated with one another and are associated with tumor metastasis and a poor patient prognosis. The data we have obtained, demonstrating shorter tumor latency in ErbB2/S14 mice compared to ErbB2 mice, is consistent with the observation made in human tumors. This is the first study to describe a link between S14 and tumor latency in a mouse model of tumorigenesis.

Preserved tissues include the tumors, the non-tumor bearing normal glands, and the lungs from all animals. The tumors will be used in various analyses, including protein, mRNA, metabolomics and microarray studies, described in the Project Plan. We have begun analyzing basic signaling pathways in the tumors collected to date. This analysis includes immunoblot evaluation of phospho-ErbB2 (Y877), total ErbB2, phospho-ErbB3 (Y1289), total ErbB3, phospho-EGFR (Y875), total EGFR, phospho-Src (Y416),

phospho-Akt (T308), and cyclin E (Figure 2). This analysis is preliminary and our goal is to screen all tumors for these molecules to get an overall picture of the signaling and proliferative events within the tumor. Other ongoing studies include immunohistochemical analyses of cleaved-caspase 3, which reflects apoptosis in the tumor, and Ki67 and BrdU, which reflect proliferation. So far, we have not seen dramatic differences in signaling molecule activation in the established tumors from different groups, and it is clear that, within a transgenic group, the activation of these pathways is quite variable. Based on the shortened tumor latency in the ErbB2/S14 mice, we evaluated the non-tumor bearing normal mammary glands using whole mount techniques for the formation of pre-neoplastic lesions. We have found that normal mammary glands from ErbB2/S14 mice display dramatic epithelial branching and budding compared to those from ErbB2 mice (Figure 3). This data is intriguing and suggests that the presence of S14 may influence the very early steps of tumorigenesis, explaining the accelerated tumor formation. Based on these observations, we will begin analyzing the same signaling pathways in the non-tumor bearing glands taken from mice to determine where S14 is influencing cell proliferation. This analysis will be conducted in addition to analysis of metabolism in tumors. We have yet to determine if the tissues or tumors display elevated lipid metabolism, or if enhanced metabolism is sufficient to promote tumor formation in the context of ErbB2 overexpression. These studies were anticipated to take place as part of Task 3 in Year 3; however, because of the sequential nature of the breeding study, these analyses have already begun and will continue through the remainder of the grant period.

As indicated previously, we have harvested lung tissue from all tumor-bearing mice to determine if S14 enhances tumor metastasis. The method we have chosen to use for analyzing lung metastasis is to preserve the entire lung tissue by injecting 10% formalin into the trachea to fill the lungs, then removing them and preserving them in cassettes in 10% formalin. After embedding the preserved tissue in paraffin, each lung is sectioned through entirely. Specifically, one section is cut, stained with H&E for visualization, then a second serial section is cut and left blank for immunostaining, and then 50 um of tissue are discarded. This pattern is continued through the entire tissue block. After consulting a pathologist, Dr. Paul Jedlicka at the University of Colorado, we feel that this method is the most reliable way to confirm lung metastases in our model. I have met with Dr. Jedlicka, who has viewed sections of lungs from our mice with me, and he has instructed me about how to identify lesions that may be mammary tumor lung metastases, as opposed to inflammatory regions, which are common in lungs of these mice. In addition, he will be available to advise me for the remainder of the study on identifying and quantitating lung metastases.

Task 3 (Year 3 Months 1-6): The tumor and other tissue samples will be analyzed using qPCR, western blot, MRS, and immunohistochemistry. These analyses will also help us

determine the effect of Spot14 on tumor cell metastasis, as lung tissues will be evaluated for the presence of mammary cancer cells. We anticipate completing this task by the second quarter of the third year.

As described above, portions of Task 3 have already been completed. We have begun analysis of tumor tissue using western blot/immunoblot and will continue with the described analyses as we obtain more samples. As part of another study in our laboratory, we have developed and optimized rapid quantitative methods to identify lipid metabolites in both cultured cells and now in whole tumor tissues using MRS and mass spectrometry. We do not anticipate any problems completing the metabolomics portion of this proposal once a sufficient sample set is obtained.

***From SOW: Specific Aim 3**: Determine the changes in the expression metabolic enzymes affected by gain or loss of Spot14 function in mammary tumors using microarray analysis.*

Task 1 (Year 1 Months 9-12): We will perform cDNA microarray analysis on tumor samples from the xenotransplantation of S14-/- and WT transformed mammary epithelial cells described in specific aim 1. We will also determine which pathways are affected by analyzing changes in the expression of specific genes associated with tumor cell metabolism. We anticipate completing this task by the last quarter of the first year.

The objective of this study is to use cDNA microarray technology to uncover novel pathways altered in tumors arising in bitransgenic mice, compared to controls. Our research to date has focused primarily on lipid metabolism, and secondarily on glucose metabolism. We may find other pathways to be differentially represented in tumors as a result of S14 expression, and we cannot eliminate the possibility that S14 has roles other than regulating lipid metabolism. Under Specific Aim 1, I described the studies completed to date for the xeno-transplant portion of this proposal. We have not obtained tissue for cDNA microarray analysis yet to complete Task 1. Studies are ongoing for this Specific Aim.

Task 2 (Year 3 Months 1-12): We will perform cDNA microarray analysis on tumor samples from transgenic mice expressing ErbB2, Spot14, or both ErbB2 and Spot14 in the mammary epithelium. This will allow us to identify changes in the expression of specific metabolic pathways modulated by Spot14 using bioinformatics analysis. We anticipate completing this task and this aim by the last quarter of the third year.

We do not anticipate any changes in the completion time for this task. Studies are ongoing and samples are currently being collected for this Specific Aim.

Key Research Accomplishments

- Cell lines have been established from S14-/- mice on the C57Bl/6 background, transformed with the PyMT antigen, and from the FVB background, transformed with the Neu oncogene.
- ACURO approval has been obtained for all animal studies for the duration of the award period.
- A mouse model has been established to test the effect of S14 overexpression on tumorigenesis and tumor metastasis and metabolism in MMTV-ErbB2 mice, and characterization of these mice has begun.
- We have established that S14 expression in mammary glands from MMTV-ErbB2 mice significantly shortens tumor latency.
- Methods have been developed and optimized to perform MRS and mass spectrometry analysis on tumor tissue, allowing us to investigate lipid metabolites in tumors.

Reportable Outcomes

Presentations:

1. Shortly after this funding period began, in November 2010, I presented the overview and Specific Aims of the proposal at our Pathology Research in Progress Seminar series. I received helpful feedback on the study design and was able to communicate with other investigators using similar models (the MMTV-ErbB2 mouse model) for their research.

2. A poster presentation was given at the DoD Era of Hope 2011 meeting in Orlando, Florida in August 2011. The abstract for that meeting is copied below.

Cancer and normal cell metabolism differ in the rate of glucose transport and glycolysis. Less well appreciated are the high rates of de novo lipid synthesis displayed by many tumors. Two related prognostic indicators are fatty acid synthase (FASN) and thyroid hormone responsive protein Spot14 (S14). Much is understood regarding the regulation and function of FASN, but little is known about the contribution of S14 to the lipogenic phenotype.

The role of S14 in lipid synthesis was determined using normal animal models. S14-/- mice experience a lactation defect characterized by reduced fatty acid synthesis. Our data indicate that S14 overexpression in mammary epithelial cells (MECs) promotes the formation of larger epithelial cytoplasmic lipid droplets during pregnancy compared to control mice. In addition, we have used mass-spectrometry (mass-spec) to determine that S14 loss in MECs from lactating dams leads to a 50% reduction in de novo synthesized short- and medium-chain lipids.

Although elevated S14 levels in breast cancer correlate with a poor prognosis, the behavior of tumors with S14 is not well characterized in vivo with regard to tumor growth rate, tumor metastasis, and alterations in metabolism. Characterizing the requirement for S14 in these tumor properties will validate its potential as a therapeutic target for breast cancer.

Based on published studies examining the role of S14 in de novo lipid synthesis, and on its association with aggressive breast cancers, we hypothesize that S14 loss will reduce growth and lipid synthesis in breast tumors and that S14 can directly stimulate de novo lipid synthesis and promote growth of mammary tumors in MMTV-c-ErbB2 mice. We are testing our hypothesis using the following studies.

Tumor growth and metabolic properties will be evaluated using a nude mouse xenograft model with wild type and S14 null mammary cancer cells. In addition, the effect of S14 overexpression on mammary tumor growth and metastasis will be evaluated using the Neu-driven mammary tumor mouse model. Once the tumor studies are completed, we will perform microarray analyses to determine which pathways and gene categories are altered by loss or gain of S14. These studies will shed light on the

mechanism through which S14 regulates lipid synthesis in breast cancer, which will define pathways involved in tumor metabolism, and will advance our understanding of a characteristic that contributes to a poor prognosis and decreased survival of patients battling the disease.

Reagent Development:

1. We have established *cell lines*, transformed using PyMT and the Neu oncogene, from S14-/- mammary glands. These lines will be useful for my studies, and also for various studies attempting to investigate the consequence of S14 loss in mammary tumor cell growth and metabolism.

2. We have generated a *mouse model* to test the effect of S14 expression on mammary tumorigenesis. This model expresses both S14 and ErbB2 in the mammary epithelium.

Conclusions

The objective of this proposal is to develop models that show a causative link between S14 and tumorigenesis, tumor metabolism and tumor metastasis. Based on the association between S14 and a poor prognosis in human tumors, and on the role of S14 in de novo lipid biosynthesis, elucidated from normal developmental models, we hypothesized that S14 loss would delay tumor growth and reduce cell metabolism and tumor metastasis. Conversely, we hypothesized that overexpression of S14 in the ErbB2 model of tumorigenesis would promote tumor formation and enhance cancer cell metabolism and metastasis to the lungs. In Year 1, we have developed a model to test our hypothesis regarding S14 overexpression. We have generated bi-transgenic mice expressing ErbB2 and S14 in the mammary epithelium. As controls, we are using mice expressing ErbB2 only, which have been widely used and are well characterized. Using this model, we have demonstrated a shorter latency in bitransgenic mice compared to controls. Furthermore, we have observed enhanced mammary epithelial branching and budding in non tumor-bearing glands in bitransgenic mice compared to controls. In the signaling molecules investigated so far, we have not observed differential activation when looking at the established tumors. Based on the morphology of the surrounding normal tissue and the non tumor-bearing glands, we predict that proliferative signaling will be different in bitransgenic mice, which could explain the shortened latency of tumor formation. Now that we have obtained a sufficient group of tumor and tissue samples, we can begin to investigate metabolic profiles and lipid synthesis enzymes.

To evaluate the effect of S14 loss on mammary tumor formation, we proposed to use xeno-transplant studies, which involve injection of transformed S14-/- or control mammary epithelial cells into the fat pads of immunocompromised mice. We have established the cell lines necessary to begin these studies, and anticipate performing the first transplant experiments within the next 2 months.

Why is this important? Currently, cancer cell and tumor metabolism are popular areas of research since tumors tend to display altered glycolysis and lipid synthesis. These alterations are generally thought to be characterized by higher metabolic activity in tumors, yet it is still unclear how tumor cells adapt their metabolic pathways. So far, in human tumors, many correlations have been made between metabolic enzymes and patient prognosis. This is an important step, but before scientists begin targeting these molecules and pathways, it is critical that we understand why the correlations exist. If we don’t causatively link metabolic proteins with an aggressive or metastatic phenotype, then it seems wasteful to develop therapies targeting these factors. Let’s use S14 as an example. In the lactating mouse mammary gland, S14 is highly expressed. Studies in our lab have focused on its role in lipid synthesis, and we have investigated the consequence of S14 loss on mammary lipid synthesis during lactation. We have found that, in S14-/- mice, there is a minor defect in the ability of the gland to synthesize lipids

from glucose, which makes S14 *necessary* for robust de novo lipid biosynthesis under these circumstances. The mammary gland, however, has evolved ways to make sure that losing one metabolic factor does not render the dam unable to feed her pups, so the uptake of pre-formed fatty acids from the diet and from adipose stores increases slightly in the gland to compensate for this. Mouse neonates are so dependent on lipids for growth and development that the gland has back-up measures in place, in case a specific critical factor fails, such as in the S14-/- mice.

If we apply this scenario to cancer, it then seems premature to draw any conclusion about S14 and tumor metabolism and metastasis, even though links between these things have been published. The goal of cancer research is to ultimately develop ways to prevent and or treat the disease. This is why basic researchers spend decades and millions of dollars trying to understand how a system functions, so that we can understand how it malfunctions. Before any therapeutic targets are aimed at any metabolic pathway or enzyme, we must understand how the pathway members fit together and regulate the phenotype of the cell or tissue. Also, we must be able to develop models that we can manipulate. We may find that targeting one molecule will not eliminate the disease, but rather create a new disease by forcing an adaptation. We need to understand if what we are observing is correlative or causative. We are hoping from these studies, to determine that S14 presence *causes* robust tumor cell proliferation and enhances metabolism. This way, we can feel more confident developing therapies that target S14 and the metabolic pathways influenced by it. Secondarily, we are aiming to link lipid metabolism in primary tumors to tumor metastasis. This is not the primary goal of the research, but it is an area of opportunity. Right now, all we know is that tumors display altered metabolism, and we know how these alterations are basically characterized. This area of research is rich with possibilities of model development and intervention, and is understudied, in my opinion. Future work in our laboratory, based on this proposal, will surely aim to investigate other molecules and links between metabolic deregulation, cancer cell signaling, and tumor growth and metastasis.

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Supporting Data

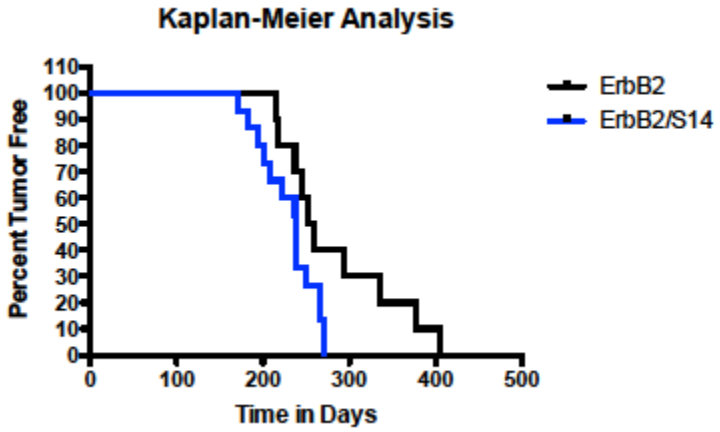
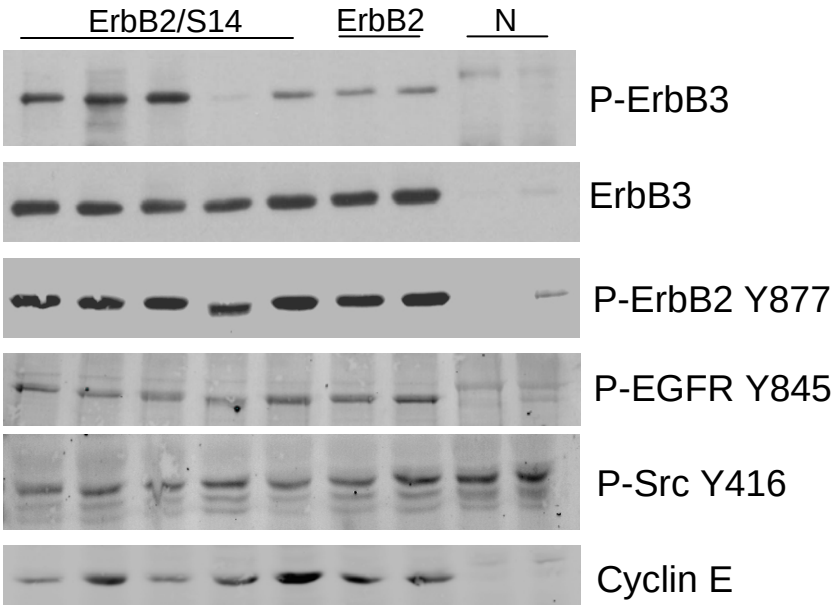


Figure 1. Preliminary Survival Analysis of Single (ErbB2) and Bitransgenic (ErbB2/S14) Mice. A Kaplan-Meier analysis was performed on all of the animals to date that have been sacrificed. This analysis does not include the animals in the study that are still alive. The study is ongoing, so these results will likely change once the last animal is sacrificed. At this point in the study the ErbB2/S14 mice have a significantly accelerated tumor latency ($p=0.038$ using the Log-Rank test). Median time to tumor onset for ErbB2 is 256 days, and for ErbB2/S14 is 238 days. Sample size for ErbB2 = 10 and for ErbB2/S14 = 15.

A.



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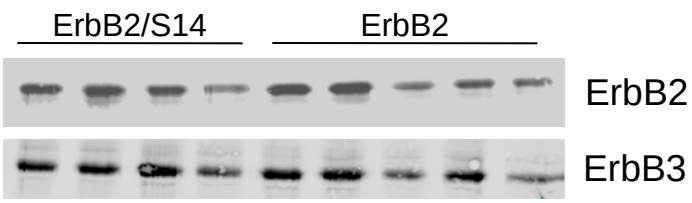

Figure 2. Western Blot Analysis of Tissues from Single (ErbB2) and Bitransgenic (ErbB2/S14) Mice. *A.* Tumors are analyzed initially in groups, making sure that several tumors from each genotype are assayed on one membrane. Immunoblot analyses show variability in Phospho-ErbB3 (P-ErbB3), and in Cyclin E. *B.* Immunoblot analysis of another group of single and bitransgenic tumors. 50 ug of protein was used for each sample. N stands for Normal Tissue. All lanes are separate tissue/tumor samples.

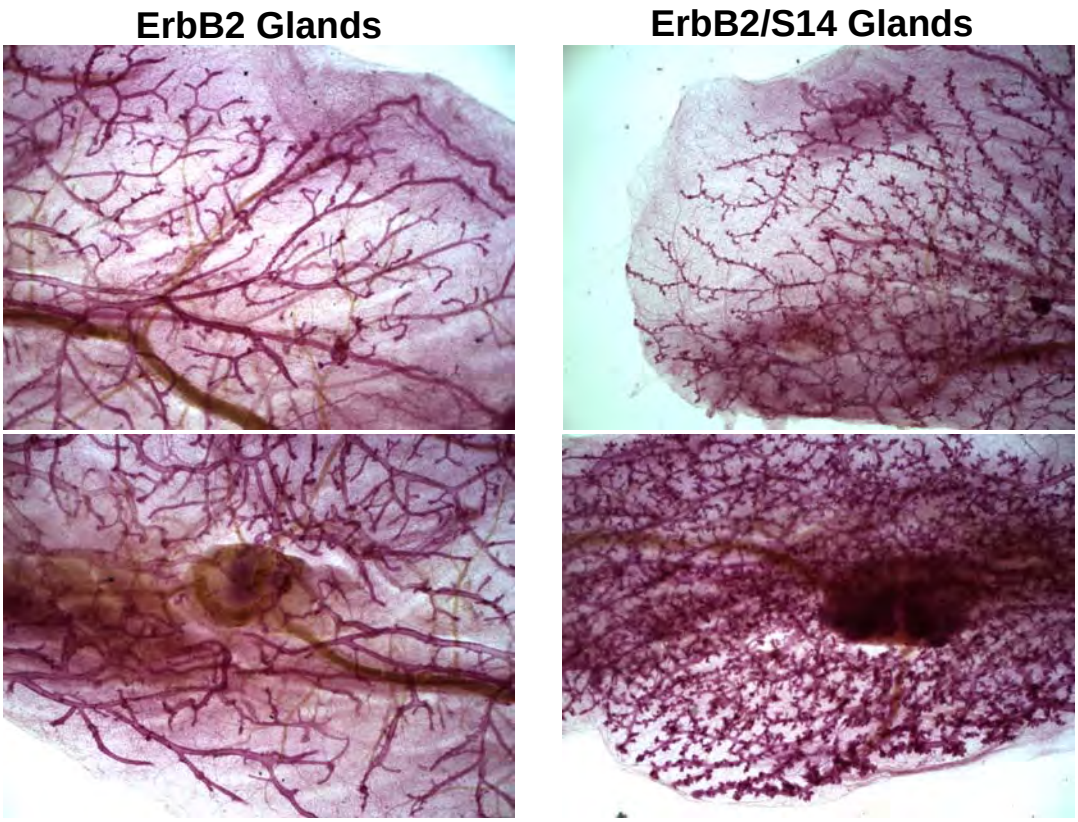

Figure 3. Mammary Gland Whole Mounts of Single (ErbB2) and Bitransgenic (ErbB2/S14) Tissues. Mammary glands were harvested from tumor-bearing mice, fixed overnight in 10% formalin and stained with Carmine-Alum solution. Note the dense epithelial budding in bitransgenic tissues compared to single transgenic tissues.